Supplementary Online Material for

Potent Inhibition of Heterotopic Ossification by Nuclear Retinoic Acid Receptor γ Agonists

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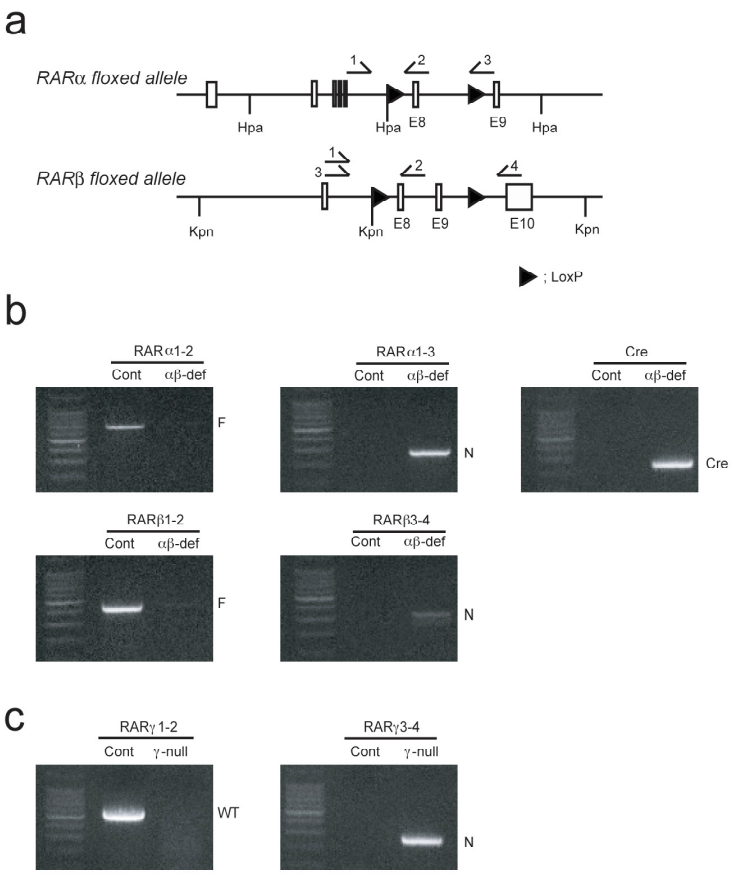
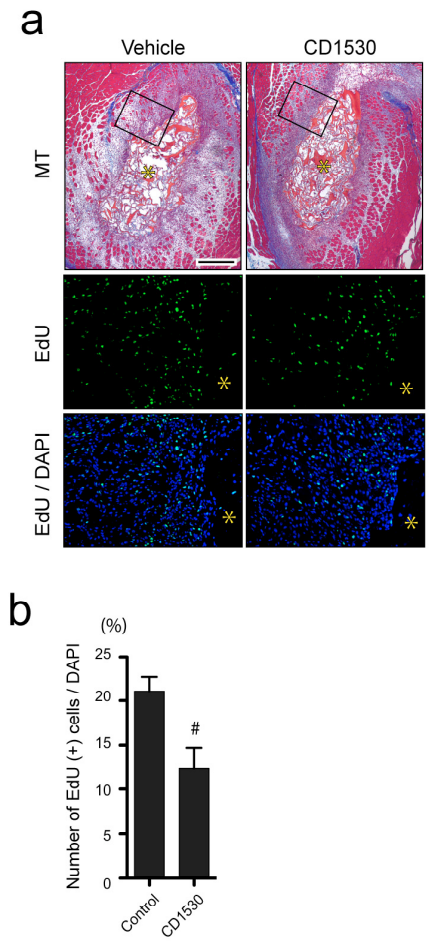
Supplemental Figures

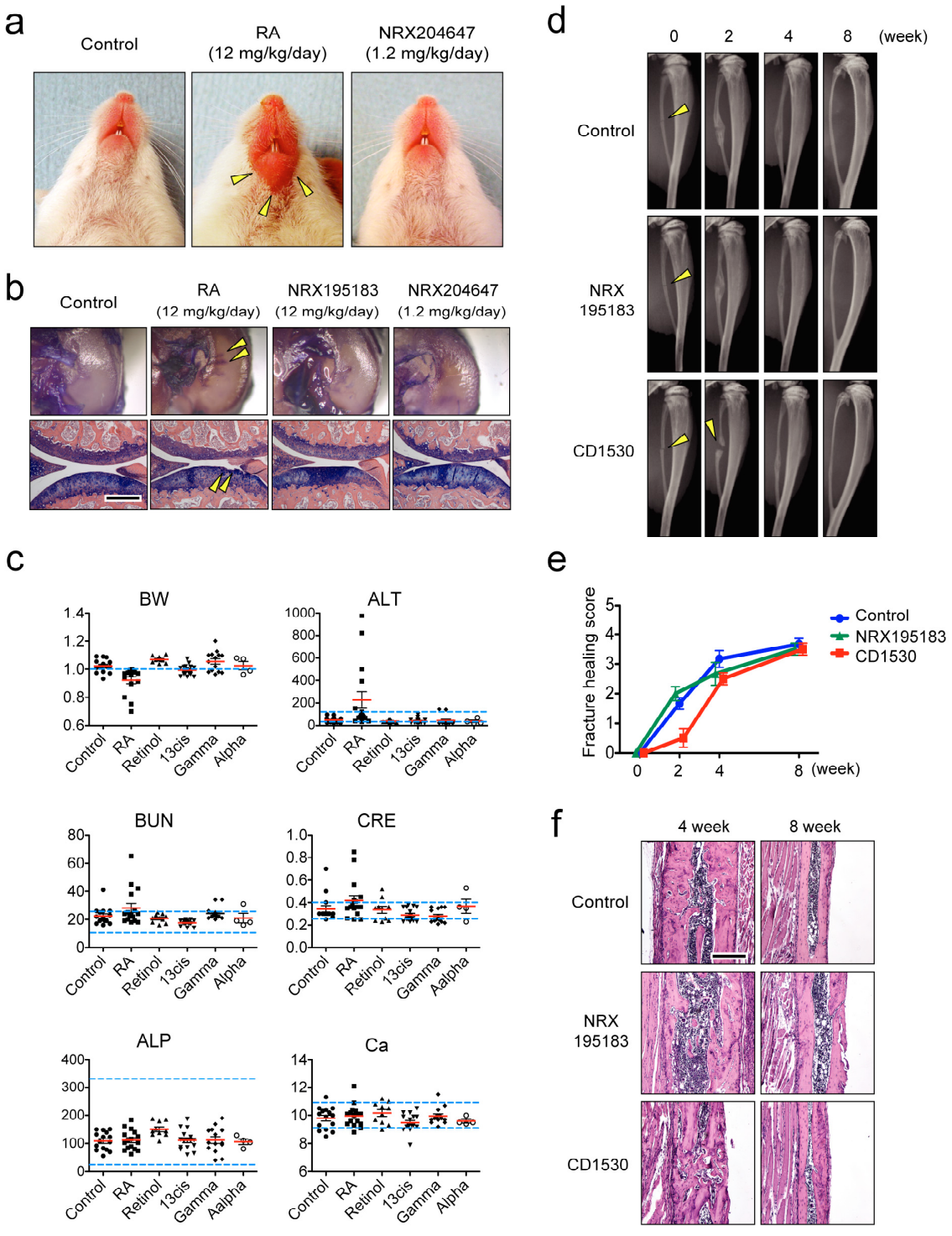


Supplementary Figure 1. Genotyping and isolation of RAR mutant limb bud cells. (**a**) Structure of floxed RARα and RARβ mouse alleles and location and direction of primers used for genotyping. (**b**) To prepare dual RARα/RARβ-deficient cells, we mated Prx1-Cre/RARα$^{f/+}$/RARβ$^{f/f}$ mice with RARα$^{f/f}$/RARβ$^{f/f}$ mice. Pregnant mice were sacrificed at E11.5, and tail DNA from each embryo was quickly processed for genotyping while the reminder the embryos were kept in serum-free medium on ice. Limb buds were then isolated from dual RARα/RARβ-deficient embryos (Prx1-Cre/RARα$^{f/f}$/RARβ$^{f/f}$) and control double floxed littermates (RARα$^{f/f}$/RARβ$^{f/f}$). Pooled limb buds were digested with 1U/ml Dispase (Dispase II, Roche Applied Science) to remove epithelium and then further digested to single cells by 0.05% trypsin-EDTA to obtain the mesenchymal cell population. One million cells of each cell pool were used to confirm Cre-mediated recombination of both RARα and RARβ genes. The remaining cells were used to prepare high-density micromass cultures. (**c**) PCR genotyping of RARγ null and control littermate limb bud cells. PCR conditions and primers were described previously*.

* Williams, J. A. *et al.* Retinoic acid receptors are required for skeletal growth, matrix homeostasis and growth plate function in postnatal mouse. *Dev. Biol.* **328**, 315-327 (2009).

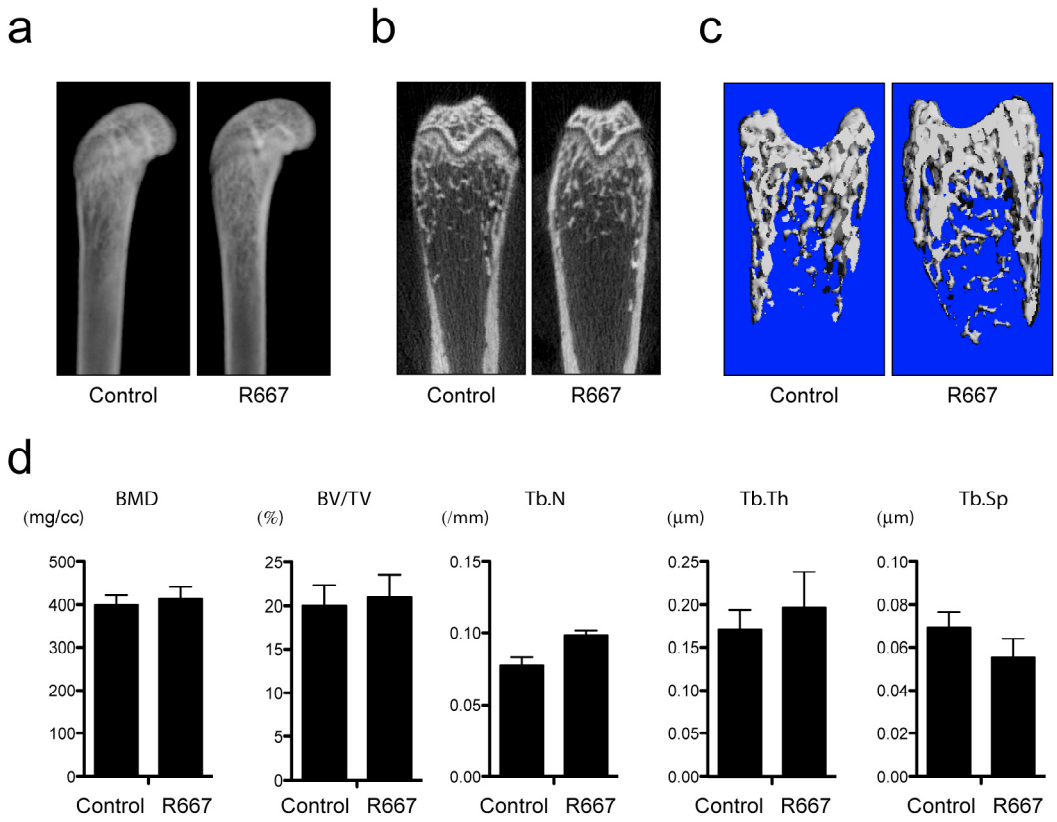


Supplementary Figure 2. Analysis of proliferative cells. (**a**) Mice implanted with rBMP-2/collagen mixture and receiving vehicle or CD1530 (4 mg/kg/day) were injected with EdU (5-ethynyl-2-deoxyuridine, Invitrogen, 100 μg/mouse) on day 4 from implantation and sacrificed on day 5. Serial sections of ectopic masses were stained with Masson's trichrome (MT) (top panel) or used for EdU detection followed by DAPI counterstaining (bottom two panels). For EdU detection, sections were de-masked by treatment with 0.1% pepsin in 0.02 N HCl for 20 min at 37°C and incorporated EdU was detected using a Click-iT EdU Alexa Flour 488 imaging kit (Invitrogen); sections were then counterstained with DAPI. In controls, MT staining (top panel) reveals the presence of cartilaginous tissue and also fibroblastic cells at the periphery of the ectopic masses (boxed area). Analysis of EdU staining in corresponding peripheral area (bottom two panels) shows presence of numerous proliferative cells. In sections from CD1530-reated mice (right column panels) cartilage is virtually absent and the number of proliferative cells is much lower. *: Collagen sponge, Bar = 1mm. (**b**) To quantify cell proliferation, the ratios of EdU-positive cells versus total DAPI-stained cells were calculated using Image J (NIH). The histogram shows the averages of ratios from 32 randomly chosen fields (8 fields per sample, n=4) in control and CD1530-treated samples. Statistical significance was determined by unpaired two-tailed *t-test* ($p < 0.05$).

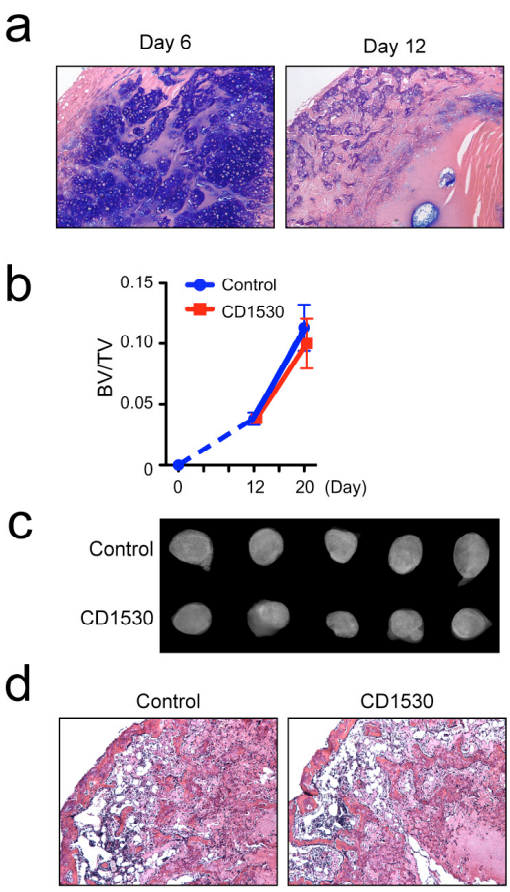


Supplementary Figure 3. Tests of side effects. (**a**) Pictures of the orofacial region of mice that had received vehicle control, RA or NRX20467 at indicated doses for 2 weeks. Note that while the NRX204647-receiving mice are essentially indistinguishable from controls, the RA-receiving mice have redness around their mouth and exhibit a loss of whiskers (arrowheads). (**b**) India ink (top row) and Alcian blue (lower row) staining of

knee joints and sections from control mice and mice treated with RA or RARγ agonists. The joints of RA-treated mice exhibit fibrillation revealed by India ink staining and articular surface defects revealed by discontinuous Alcian blue staining (arrowheads). This was expected because high doses of RA are known to shift the homeostatic mechanisms operating in cartilage and synovial joints toward extracellular matrix degradation. However, the joints of RARγ agonist-treated animals closely resemble those in controls and have no obvious defects. (**c**) Body weight (BW) measurements and VetScan peripheral blood maker analyses showing that RA-treated mice had lost some weight and had elevated values of creatinine (CRE), urea nitrogen (BUN) and alanine aminotransferase (ALT), indicative of kidney and liver function changes. Other values including alkaline phosphatase (ALP) and total calcium (Ca) did not change. (**d-f**) Tests of bone fracture healing. While HO is a pathological process, formation of endochondral bone is normally needed for fracture repair, and it is possible that certain patients in danger of developing HO, such as severely wounded soldiers, could have broken bones as well. To assess possible side effects on fracture repair, we used a relatively benign fracture repair model in which the mouse fibula is transected and allowed to heal without stabilization (**d**). Following surgery, mice were divided into groups and received the RARγ agonist CD1530 (12 mg/kg/day), the RARα agonist NRX191583 (36 mg/kg/day) or vehicle for 3 weeks by gavage. Fracture healing was then monitored over time in the same animals by soft x-ray for a grand total of 8 weeks (**d-e**) and was further verified by histology at 4- and 8-week time points (**f**). The data show that mice receiving the RARα agonist healed their fracture as well and as readily as control untreated mice, but there was a transient delay in fracture healing at 2 weeks in mice receiving the RARγ agonist that was fully overcome by 4 and 8 weeks.



Supplementary Figure 4. Analyses of bone structure and density. (**a-c**) Long bones in mice receiving vehicle or RARγ agonist R667 (4.0 mg/kg/day) were examined by: (**a**) soft x-ray; (**b**) two dimensional μCT; and (**c**) three dimensional μCT. Data were used to determine bone mineral density (BMD), BV/TV, Tb.N, Tb.Th and Tb.Sp. No major differences in these parameters are appreciable between control and treated mice.



Supplementary Figure 5. Analysis of retinoid effects on the osteogenic stage of HO development. Mice implanted with standard rBMP2/Matrigel mixture subcutaneously were left untreated until day 12 and then received vehicle or CD1530 (12 mg/kg/day) until day 20. (**a**) Histology on samples of ectopic tissues taken from some of the mice at day 6 and day 12 from implantation show that cartilage was present at day 6 while bone was predominant by day 12. (**b**) Bone volume/total tissue volume ratios show that the levels of mineralized tissue after day 12 continued to increase similarly in both control and treated mice. (**c-d**) Soft x-ray images and histological images of ectopic masses isolated from 5 control and 5 CD1530-treated mice at day 20 confirm that there were no major differences in ectopic bone tissue in control and treated mice.

Supplementary Methods

Histological and immunohistochemical analyses. Skeletal samples were decalcified in 10% EDTA and embedded in paraffin. Continuous 5 μm-thick sections were stained with hematoxylin and eosin, Masson's trichrome or Alcian blue at pH 1.0. Alcian blue-stained sections were counterstained with eosin. Tartrate-resistant acid phosphatase (TRAP) staining was performed using a leukocyte acid phosphatase kit (Sigma) and sections were counterstained with methyl green. For immunohistochemistry, sections were reacted with one of the following antibodies against: myosin heavy chain (Developmental Studies Hybridoma Bank, MF-20, 1: 25) detected with goat anti-mouse Alexa Flour 594 IgGs (Invitrogen, A11005, 1:500); osteocalcin (Takara, M173, 1:500) detected with goat anti-Rabbit Alexa Flour 594 IgGs (Invitrogen, A11012, 1:500); or biotin conjugated anti-GFP (Novus, NB100-1678, 1: 250) detected with streptavidin Alexa Flour 488 (Invitrogen, S11223, 1:500)

Dual reporter assays. The skeletogenic cell line ATDC5 cells and mouse MSCs were seeded in monolayer culture and serum-starved overnight. Cultures were co-transfected with 100 ng per dish of canonical BMP signaling reporter Id1-Luc plasmid and 1 ng/dish of phRG-TK (Promega) using 0.2 μl of Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. Twenty-four hours later, cells were harvested and subjected to dual luciferase assay (Promega); transfection efficiency was normalized to Renilla luciferase activity generated by phRG-TK.

Protein analysis. ATDC5 cells were grown to about 70% confluence in 6 well plates and treated with indicated concentrations of rBMP-2 and/or RARγ agonists. Total cellular proteins harvested in SDS-PAGE sample buffer were electrophoresed on 4-15% SDS-bis-Tris gels (40 μg per lane) and transferred to PVDF membranes (Invitrogen). Membranes were incubated overnight at 4°C with dilutions of antibodies against Smad1 (Epitomics, 1694-1, 1:1000), phospho-Smad1/5/8 (Cell Signaling Technology, 9511, 1:1000), Smad3 (Cell Signaling Technology, 9513, 1:500), Smad4 (Cell Signaling Technology, 9515, 1:500) or Smad5 (Cell Signaling Technology, 9517, 1:1000). Enhanced chemioluminescent immunoblotting detection system (Pierce) was used to detect the antigen-antibody complexes. The membranes were re-blotted with antibodies to α-tubulin (Sigma, T-5168, 1:2000) for normalization, and band intensities were quantified by computer-assisted image analysis.

Immunocytochemistry. Chondrogenic cells were seeded onto type 1 collagen-coated culture slides (BD Bioscience) at a density of 1×10^4 cells per well and maintained in 10% FBS in DMEM/F12 (Cellgro) overnight. Cells were then switched to 0.3%FBS in DMEM/F12 for 24 hrs and treated with 100 ng ml^{-1} of rBMP-2 or combination of rBMP-2 and 1 μM of CD1530. After 2 hrs, cells were fixed with 4% neutralized formalin, permeabilized with acetone/ethanol mixture (50:50, v/v) at -20°C for 1 min, and incubated with primary rabbit anti-Smad1 polyclonal antibodies (Epitomics, 1694-1, 1:250) overnight at 4°C followed by incubation with goat anti-Rabbit Alexa Flour 594 IgGs (Invitrogen, A11012, 1:500) for 30 min. In some experiments, cultures were also stained with 0.1 μg ml^{-1} DAPI (Sigma) for nuclear staining or with Alexa fluor 488-

phalloidin (Invitrogen, A12379, 1:500) for cytoplasmic β-actin staining. Samples were mounted with GEL/MOUNT (Biomeda) and viewed with a fluorescent microscope (Eclipse TE2000-U, Nikon).